

Attorney Docket No: 02312/2082B (formerly 1440.1027-016)  
U.S. App. No. 10/032,221)  
Inventor: Raghuram Kalluri  
Filed: December 21, 2001  
Sequence Listing and Preliminary Amendment  
Page 2

Transmitted herewith is a copy of the "Sequence Listing" (sheets 1/27 through 27/27) in paper form for the above-identified patent application as required by 37 C.F.R. §1.821(c) and a copy of the Sequence Listing in computer readable form as required by 37 C.F.R. §1.821(e). As required by 37 C.F.R. §1.821(f), Applicant's Attorney hereby states that the content of the "Sequence Listing" in paper form and the computer readable form of the "Sequence Listing" are the same and, as required by 37 C.F.R. §1.821(g), also states that the submission includes no new matter.

Applicants Attorney submits the following amendments to comply with 37 C.F.R. §1.825:

#### **AMENDMENTS**

##### **In the specification:**

Please insert the attached "Sequence Listing" (sheets 1/27 through 27/27, and comprising SEQ ID NOs:1-58, into the above-referenced application.

Please replace the paragraph at page 5, lines 11 through 17 with the following paragraph:

The invention also relates to an isolated mutated fragment of SEQ ID NO:10, where one to five amino acids have been substituted, and where the mutated fragment has the ability to inhibit angiogenesis. The fragment can be T7-mutant (SEQ ID NO:38), T8 (SEQ ID NO:39), T8-3 (SEQ ID NO:40), TP3 (SEQ ID NO:41) or P2 (SEQ ID NO:42). Such fragments can also be reduced, alkylated, or oxidized. Such fragments can also have one or more of the cysteine residues substituted for another amino acid.

Please replace the paragraph at page 5, lines 18 through 24 with the following paragraph:

The invention further relates to an isolated fragment of SEQ ID NO:10, which has the ability to inhibit protein synthesis in endothelial cells. The fragment can be T7 (SEQ

ID NO:37), T7-mutant (SEQ ID NO:38), T8 (SEQ ID NO:39), T8-3 (SEQ ID NO:40), TP3 (SEQ ID NO:41) or P2 (SEQ ID NO:42). Such fragments can be reduced, alkylated, or oxidized. Such fragments can also have one or more of the cysteine residues substituted for another amino acid. The protein synthesis can be cap-dependent protein synthesis. The cells can express the  $\alpha_v\beta_3$  integrin.

Please replace the paragraph at page 6, lines 4 through 14 with the following paragraph:

In an additional aspect, the invention relates to a method for inhibiting angiogenic activity in mammalian tissue, where the method includes contacting the tissue with a composition containing an isolated fragment selected from the group consisting of: (a) SEQ ID NO:10; (b) amino acid 1 through amino acid 124 of SEQ ID NO:10; (c) SEQ ID NO:20; (d) SEQ ID NO:21; (e) SEQ ID NO:22; (f) SEQ ID NO:23; (g) SEQ ID NO:25; (h) SEQ ID NO:26; (i) SEQ ID NO:29; (j) SEQ ID NO:30; (k) SEQ ID NO:33; (l) SEQ ID NO:34; (m) SEQ ID NO:37; (n) SEQ ID NO:38; (o) SEQ ID NO:39; (p) SEQ ID NO:40; (q) SEQ ID NO:41; and/or (r) SEQ ID NO:42. Such fragments can also be reduced, alkylated, or oxidized. Such fragments can also have one or more of the cysteine residues substituted for another amino acid.

Please replace the paragraph at page 6, lines 15 through 25 with the following paragraph:

In an additional aspect, the invention relates to a method for inhibiting tumor growth in mammalian tissue, where the method includes contacting the tissue with a composition containing an isolated fragment selected from the group consisting of: (a) SEQ ID NO:10; (b) amino acid 1 through amino acid 124 of SEQ ID NO:10; (c) SEQ ID NO:20; (d) SEQ ID NO:21; (e) SEQ ID NO:22; (f) SEQ ID NO:23; (g) SEQ ID NO:25; (h) SEQ ID NO:26; (i) SEQ ID NO:29; (j) SEQ ID NO:30; (k) SEQ ID NO:33; (l) SEQ ID NO:34; (m) SEQ ID NO:37; (n) SEQ ID NO:38; (o) SEQ ID NO:39; (p) SEQ ID

NO:40; (q) SEQ ID NO:41; and/or (r) SEQ ID NO:42. Such fragments can also be reduced, alkylated, or oxidized. Such fragments can also have one or more of the cysteine residues substituted for another amino acid.

Please replace the paragraph at page 6, line 26 through page 7, line 9 with the following paragraph:

In an additional aspect, the invention relates to a method for inhibiting protein synthesis in one or more mammalian cells, where the method includes contacting the cells with a composition containing an isolated fragment selected from the group consisting of: (a) SEQ ID NO:10; (b) amino acid 1 through amino acid 124 of SEQ ID NO:10; (c) SEQ ID NO:20; (d) SEQ ID NO:21; (e) SEQ ID NO:22; (f) SEQ ID NO:23; (g) SEQ ID NO:25; (h) SEQ ID NO:26; (i) SEQ ID NO:29; (j) SEQ ID NO:30; (k) SEQ ID NO:33; (l) SEQ ID NO:34; (m) SEQ ID NO:37; (n) SEQ ID NO:38; (o) SEQ ID NO:39; (p) SEQ ID NO:40; (q) SEQ ID NO:41; and/or (r) SEQ ID NO:42. Such fragments can also be reduced, alkylated, or oxidized. Such fragments can also have one or more of the cysteine residues substituted for another amino acid. The protein synthesis can be cap-dependent protein synthesis. The cells can be endothelial cells. The cells can express the  $\alpha_v\beta_3$  integrin.

Please replace the paragraph at page 8, line 17 through page 10, line 2 with the following paragraph:

The invention also relates to an anti-angiogenic, isolated non-Goodpasture fragment of  $\alpha 3(IV)$  NC1 domain, which has one or more of the following characteristics: (a) the ability to bind  $\alpha_v\beta_3$  integrin; (b) the ability to inhibit proliferation of endothelial cells; and (c) the ability to cause apoptosis of endothelial cells. The isolated non-Goodpasture fragment binds  $\alpha_v\beta_3$  integrin by an RGD-independent mechanism, as

described herein. Such an isolated fragment of the  $\alpha 3(\text{IV})\text{NC1}$  domain of Type IV collagen is described herein, and is designated "Tumstatin." "Tumstatin", as the term is used herein, comprises SEQ ID NO:10. In addition, another isolated non-Goodpasture fragment, designated herein as "Tum-1", or "Tumstatin N53" (SEQ ID NO:22), consists of the amino acid sequence of amino acid residue 54 to amino acid 244 of full-length Tumstatin (SEQ ID NO:10). Other isolated fragments disclosed herein include "Tum-2" (SEQ ID NO:23), "Tum-3" (SEQ ID NO:24), "Tum-4" (SEQ ID NO:25), and "Tum-5" (SEQ ID NO:26), which consist of the amino acid sequence of residues 1 to 132 (Tum-2), residues 133 to 244 (Tum-3), residues 181 to 244 (Tum-4), and residues 54 to 132 (Tum-5) of full-length Tumstatin (SEQ ID NO:10), respectively. Peptide fragments are also disclosed herein, including "T1" (SEQ ID NO:27), "T2" (SEQ ID NO:28), "T3" (SEQ ID NO:29), "T4" (SEQ ID NO:30), "T5" (SEQ ID NO:31), "T6" (SEQ ID NO:32) and "T7" (SEQ ID NO:37), which consist of amino acid residues 1 to 19 (T1), 53 to 72 (T2), 68 to 87 (T3), 83 to 102 (T4), 98 to 116 (T5), 113 to 131 (T6) and 73 to 97 (T7), respectively, of full-length Tumstatin (SEQ ID NO:10). Yet another peptide fragment of full-length Tumstatin is designated herein as "Tumstatin-45-132" (SEQ ID NO:33) and consists of amino acid residues 45 to 132 of full-length Tumstatin (SEQ ID NO:10). Another fragment of full-length Tumstatin is designated herein as "Tum-5-125-C-A" (SEQ ID NO:34), and consists of Tumstatin-45-132, where the cysteine at position 125 (of full-length Tumstatin) is mutated via site-directed mutagenesis to alanine. Fragments of Tumstatin which are reduced, *e.g.*, alkaline reduced, are also described herein to possess anti-angiogenic properties. Two other fragments are "Tumstatin 333" (SEQ ID NO:20) and "Tumstatin 334" (SEQ ID NO:21), which consist of residues 1 through 124 (Tumstatin 333) and residues 125 through 244 of full-length Tumstatin (SEQ ID NO:10). Other fragments of Tumstatin include T7-mutant (SEQ ID NO:38, methionine has been substituted for the leucine residue at position 77 of the full-length Tumstatin molecule, and isoleucine has been substituted for valine at position 81, and asparagine has been substituted for aspartic acid at position 83), T8 (SEQ ID NO:39, lysine has been

substituted for the leucine residue at position 68 of the full-length Tumstatin molecule), T8-3 (SEQ ID NO:40, in which lysine has been substituted for the leucine residue at position 68 of the full-length Tumstatin molecule, and serine has been substituted for the cysteine residues at positions 79 and 85), TP3 (SEQ ID NO:41, in which lysine has been substituted for the phenylalanine residue at position 76 of the full-length Tumstatin molecule, and cysteine has been substituted for the aspartic acid at position 83), and P2 (SEQ ID NO:42, in which lysine has been substituted for the leucine residue at position 68 of the full-length Tumstatin molecule, and and aspartic acid has been substituted for the cysteine residues at positions 79 and 85).

Please replace the paragraph at page 10, lines 3 through 20 with the following paragraph:

The invention also features an anti-tumor cell, isolated non-Goodpasture fragment of  $\alpha 3(\text{IV})$  NC1 domain, which has one or more of the following characteristics: (a) the ability to bind  $\alpha_v\beta_3$  integrin, (b) the ability to bind endothelial cells, (c) the ability to inhibit proliferation of tumor cells, and (d) the inability to inhibit proliferation of endothelial cells. The isolated non-Goodpasture fragment can bind  $\alpha_v\beta_3$  integrin by an RGD-independent mechanism, as described herein. One isolated non-Goodpasture fragment comprises the amino acid sequence of amino acid residue 185 to amino acid 203 of full-length Tumstatin (SEQ ID NO:10). Another peptide fragment of full-length Tumstatin is designated herein as "T3," and consists of amino acid residues 68 to 87 of full-length Tumstatin (SEQ ID NO:10). Yet another peptide fragment of full-length Tumstatin is designated herein as "Tumstatin-45-132," and consists of amino acid residues 45 to 132 of full-length Tumstatin (SEQ ID NO:10). Another fragment of full-length Tumstatin is designated herein as "Tum-5-125-C-A" (SEQ ID NO:34), and consists of Tumstatin-45-132 (SEQ ID NO:33), where the cysteine at position 125 (of full-length Tumstatin) is mutated via site-directed mutagenesis to alanine. Fragments of Tumstatin which are reduced, *e.g.*, alkaline reduced, are also described herein to possess

anti-angiogenic properties. Other fragments of Tumstatin include T7-mutant, T8, T8-3, TP3, and P2.

Please replace the paragraph at page 30, lines 22 through 26 with the following paragraph:

Fig. 51 is a histogram showing the effect of *E. coli*-expressed Tumstatin-45-132 and Tum-5-125-C-A on progression of the cell cycle. The percentage of C-PAE cells in S phase (y-axis) is shown at hour 0 (control), and after treatment by 0, 1, 10 and 20  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132 (black bars) or Tum-5-125-C-A (white bars). The experiments were repeated three times.

Please replace the paragraph at page 30, line 27 through page 31, line 8 with the following paragraph:

Figs. 52A, 52B, 52C and 52D are a set of four histograms showing the effects of Tumstatin-45-132 and Tum-5-125-C-A on cell viability. Fig. 52A shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for C-PAE cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132 (black bars) and Tumstatin-45-132 that was alkylated and reduced (white bars). Fig. 52B shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for C-PAE cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tum-5-125-C-A. Fig. 52C shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for PC-3 cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132. Fig. 52D shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for DU-145 cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132.

Please replace the paragraph at page 31, lines 13 through 17 with the following paragraph:

Fig. 54 is a line graph showing the fractional tumor volume (y-axis) in terms of  $V/V_0$  (mean tumor volume/initial tumor volume) at 0, 5, 10, 15 and 20 days (x-axis) of treatment with vehicle (control,  $\square$ ), 1 mg/kg Tumstatin-45-132 ( $\diamond$ ), 1 mg/kg Tum-5-125-C-A ( $\bullet$ ), 20 mg/kg endostatin ( $\circ$ ) and mini-pump administered Tumstatin-45-132 (1 mg/kg,  $\Delta$ ).

Please replace the paragraph at page 46, line 22 through page 47, line 8 with the following paragraph:

Besides Tum-1, other Tumstatin deletion mutants were also created, including Tum-2, Tum-3 and Tum-4. These are also described in Example 35, below. Tum-1, as stated above, comprises the C-terminal 191 amino acids, and is lacking the N-terminal 53 amino acids. "Tumstatin 333" comprises the N-terminal amino acids 1 to 124 of Tumstatin. Tum-3 comprises the C-terminal 112 amino acids. Tum-4 comprises the C-terminal 64 amino acids, which includes amino acids 185-203 (Han *et al.*, 1997, *J. Biol. Chem.* 272:20395-401). The region of amino acids 54 to 132 of full-length Tumstatin was designated Tum-5. An extended version of Tum-5, designated herein as "Tumstatin-45-132", was created to increase the expression and solubility of Tum-5. Tumstatin-45-132 consists of Tum-5, with an extension at the N-terminal end of an additional nine amino acids. In addition, a mutant of Tumstatin-45-132 was created, designated herein as "Tum-5-125-C-A". This mutant consists of the sequence of Tumstatin-45-132, where the cysteine at position 125 (of full-length Tumstatin) is mutated via site-directed mutagenesis to alanine. Further deletion mutants were made of Tum-5, which comprised T1 and a set of partially overlapping peptides (T2, T3, T4, T5 and T6).

Please replace the table at page 47, line 11 through page 48, line 27 with the following table:

Protein	Residues	Size	SEQ ID NO:
Tumstatin (full-length)	<u>1</u> <u>244</u>	<u>244</u>	10
Tumstatin 333	<u>1</u> <u>124</u>	124	20
Tumstatin 334	<u>125</u> <u>244</u>	<u>120</u>	21
Tum-1 (Tumstatin N53)	<u>54</u> <u>244</u>	191	22
Tum-2	<u>1</u> <u>132</u>	132	23
Tum-3	<u>133</u> <u>244</u>	112	24
Tum-4	<u>181</u> <u>244</u>	64	25
Tum-5	<u>54</u> <u>132</u>	79	26
T1	<u>1</u> <u>19</u>	<u>19</u>	27
T2	<u>53</u> <u>72</u>	20	28
T3	<u>68</u> <u>87</u>	20	29
T4	<u>83</u> <u>102</u>	20	30
T5	<u>98</u> <u>116</u>	19	31
T6	<u>113</u> <u>131</u>	19	32
Tumstatin-45-132	<u>45</u> <u>132</u>	88	33
Tum-5- <u>125</u> -C-A	<u>45</u> <u>132</u> <sup>1</sup>	88	34
T7	<u>73</u> <u>97</u>	25	37
T7-mutant	<u>73</u> <u>97</u> <sup>2</sup>	25	38
T8	<u>68</u> <u>94</u> <sup>3</sup>	27	39
T8-3	<u>68</u> <u>94</u> <sup>4</sup>	27	40
TP3	<u>76</u> <u>94</u> <sup>5</sup>	19	41
P2	<u>68</u> <u>94</u> <sup>6</sup>	27	42

<sup>1</sup> In Tum-5-125-C-A, alanine has been substituted for the cysteine residue at position 125 of the full-length Tumstatin molecule.



<sup>2</sup> In T7-mutant, methionine has been substituted for the leucine residue at position 77 of the full-length Tumstatin molecule, and isoleucine has been substituted for valine at position 81, and asparagine has been substituted for aspartic acid at position 83.

<sup>3</sup> In T8, lysine has been substituted for the leucine residue at position 68 of the full-length Tumstatin molecule.

<sup>4</sup> In T8-3, lysine has been substituted for the leucine residue at position 68 of the full-length Tumstatin molecule, and serine has been substituted for the cysteine residues at positions 79 and 85.

<sup>5</sup> In TP3, lysine has been substituted for the phenylalanine residue at position 76 of the full-length Tumstatin molecule, and cysteine has been substituted for the aspartic acid at position 83.

<sup>6</sup> In P2, lysine has been substituted for the leucine residue at position 68 of the full-length Tumstatin molecule, and and aspartic acid has been substituted for the cysteine residues at positions 79 and 85.

Please replace the paragraph at page 50, lines 24 through 29 with the following paragraph:

A mutant of Tumstatin-45-132 was created, Tum-5-125-C-A, in which the cysteine at residue number 125 (in the full-length molecule) is mutated to alanine. This mutation exhibits enhanced protein expression, and the molecule possesses anti-angiogenic properties equivalent to Tumstatin-45-132, with the exception of inhibition of tumor growth in mouse xenograft studies, where the mutant actually inhibited tumor growth more strongly than Tumstatin-45-132.

Please replace the paragraph at page 52, lines 3 through 10 with the following paragraph:

60	65	70	75	80	85	90	95	100
Tumstatin: DLGTLGSCLQRFTTMPFLFCNVNDVCNFASRNDYSYWLSTP								
T7				TMPFLFCNVNDVCNFASRNDYSYWL				
T7-mutant				TMPFmFCNiNnVCNFASRNDYSYWL				
T8				kQRFTTMPFLFCNVNDVCNFASRNDYS				
T8-3				kQRFTTMPFLFsNVNDVsNFASRNDYS				
TP3				kLFCNVNcVCNFASRNDYS				
P2				kQRFTTMPFLFdNVNDVdNFASRNDYS				

Please replace the paragraph at page 52, lines 11 through 21 with the following paragraph:

Tumstatin peptide T7 is a fragment of full-length Tumstatin, with no alterations in the sequence. Peptide T7-mutant is based on the T7 sequence, but has methionine, isoleucine and asparagine substituting for leucine, valine and aspartic acid at Tumstatin residues 77, 81 and 83, respectively. Peptide T8 has lysine substituted for leucine at Tumstatin position 68. Peptide T8-3 has two additional substitutions, where a serine has been substituted for each of the cysteine residues at Tumstatin positions 79 and 85. Peptide TP3 has lysine substituted for the phenylalanine residue at position 76, and cysteine has been substituted for the aspartic acid at position 83. Peptide P2 is also similar to the T8-3 peptide, also having a lysine substituted for the leucine at Tumstatin position 68, but with the cysteines at positions 79 and 85 being replaced by an aspartic acid.

Please replace the paragraph at page 53, lines 3 through 13 with the following paragraph:

In a PC3 human prostate tumor xenograft model, where peptides T7, T8, TP3, and control scrambled peptide SP1 and SP2 were administered daily, T8, T7 and TP3 at 5 mg per kg inhibited PC3 tumor growth by 45%, 66.8% and 53.2%, respectively. SP1 and SP2 inhibited growth by 31.7 and 18.7%. When administered at 5 mg per kg once a week, T8 inhibited tumor growth by 39.5%, but only 8.1% when administered twice a

week, thus mirroring the results in the MDAMB-435 model. In another experiment, both the T8 and T8-3 peptides inhibited tumor growth by 35.4% at dosages of 5 mg per kg, showing that the cysteines at positions 79 and 85 do not provide a secondary structure that is required for this biological activity. P2 proved to be more effective at lower doses in the PC3 model as well as the MDAMB-435 model, inhibiting tumor growth by 31.6% and only 15.9% at 1 and 5 mg per kg, respectively.

Please replace the paragraph at page 62, lines 15 through 22 with the following paragraph:

	60	65	70	75	80	85	90	95	100
Tumstatin:	dlgtlgsc	lqrftt	mpfL	FcNVNd	VcNF	asrndys	ylstp		
T3			lqrftt	mpfL	FcNVNd	VcNF			
T7				tmpfL	FcNVNd	VcNF	asrndys	yl	
T8			kqrftt	mpfL	FcNVNd	VcNF	asrndys		
T8-3			kqrftt	mpfL	FsNVNd	VsNF	asrndys		
Tp3					kL	FcNVNc	VcNF	asrndys	
P2			kqrftt	mpfL	FdNVNd	VdNF	asrndys		

Please replace the paragraph at page 63, lines 3 through 15 with the following paragraph:

Other fragments can also be made. One fragment of Tumstatin, designated "Tumstatin N-53", was found to have anti-angiogenic activity equivalent to that of full-length Tumstatin, as determined by standard assays. Tumstatin N-53 comprises a Tumstatin molecule wherein the N-terminal 53 amino acids have been deleted. Other mutant fragments described herein have been found to have very high levels of anti-angiogenic activity, as shown by the assays described herein. These fragments,"Tumstatin 333," "Tumstatin 334," "12 kDa Arresten fragment," "8 kDa Arresten fragment," and "10 kDa Canstatin fragment" have ED<sub>50</sub> values of 75 ng/ml, 20 ng/ml, 50 ng/ml, 50 ng/ml, and 80 ng/ml, respectively. By contrast, full-length Arresten,

Canstatin and Tumstatin were found to have ED<sub>50</sub> values of 400 ng/ml, 400 ng/ml, and 550 ng/ml, respectively. Tumstatin 333 comprises amino acids 1 to 124 of SEQ ID NO:10, and Tumstatin 334 comprises amino acids 125 to 244 of SEQ ID NO:10.

Please replace the paragraph at page 67, lines 3 through 9 with the following paragraph:

Identity is often measured using sequence analysis software *e.g.*, BLASTN or BLASTP (available at the world wide web site (“www”) for the National Center for Biotechnology Information (“ncbi”) of the National Institutes of Health (“nih”) of the U.S. government (“gov”), in the “/BLAST/” directory ). The default parameters for comparing two sequences (*e.g.*, “Blast”-ing two sequences against each other) by BLASTN (for nucleotide sequences) are reward for match = 1, penalty for mismatch = - 2, open gap = 5, extension gap = 2. When using BLASTP for protein sequences, the default parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1.

Please replace the paragraph at page 68, lines 1 through 7 with the following paragraph:

The invention contemplates mutants of the proteins and peptides disclosed herein, where the mutation(s) do not substantially alter the activity of the protein or peptide, that is the mutations are effectively “silent” mutations. One such mutant, Tum-5-125-C-A, is presented herein, in which the cysteine at the 125th residue (of the full-length Tumstatin molecule) has been mutated from cysteine to alanine. This mutation prevents a disulfide bond from being formed at that residue, yet Tum-5-125-C-A retains the full activity of its parent molecule Tumstatin-45-132.

Please replace the paragraph at page 124, line 22 through page 125, line 28 with the following paragraph:

The nucleotide (SEQ ID NO:9) and amino acid (SEQ ID NO:10) sequences for the  $\alpha 3$  chain of the NC1 domain of Type IV collagen are shown in Figs. 18A and 18B, respectively. The sequence encoding Tumstatin was amplified by PCR from the  $\alpha 3$  NCI (IV)/pDS vector (Neilson, E.G. *et al.*, 1993, *J. Biol. Chem.* 268:8402-5; GenBank Accession Nos. M92993 (Quinones, S. *et al.*, 1994), M81379 (Turner, N. *et al.*, 1994), and X80031 (Leionin, A.K., and Mariyama, M. *et al.*, 1998)) using the forward primer 5'-CGG GAT CCG GGT TTG AAA GGA AAA CGT-3' (SEQ ID NO:11) and the reverse primer 5'-CCC AAG CTT TCA GTG TCT TTT CTT CAT-3' (SEQ ID NO:12). The resulting cDNA fragment was digested with *Bam*HI and *Hind*III and ligated into predigested pET22b(+) (Novagen, Madison, Wisconsin, USA). The construct is shown in Fig. 19. The ligation placed Tumstatin downstream of and in-frame with the pelB leader sequence, allowing for periplasmic localization and expression of soluble protein. Additional vector sequence was added to the protein encoding amino acids MDIGINSD (SEQ ID NO:13). The 3' end of the sequence was ligated in-frame with the polyhistidine tag sequence. Additional vector sequence between the 3' end of the cDNA and the his-tag encoded the amino acids KLAAALE (SEQ ID NO:14). Positive clones were sequenced on both strands. Plasmid constructs encoding Tumstatin were first transformed into *E. coli* HMS174 (Novagen, Madison, Wisconsin, USA) and then transformed into BL21 for expression (Novagen, Madison, Wisconsin, USA). Overnight bacterial culture was used to inoculate a 500 ml culture in LB medium (Fisher Scientific, Pittsburgh, Pennsylvania, USA). This culture was grown for approximately 4 hours until the cells reached an OD<sub>600</sub> of 0.6. Protein expression was then induced by addition of IPTG to a final concentration of 1 mM. After a 2-hour induction, cells were harvested by centrifugation at 5,000 x g and lysed by resuspension in 6 M guanidine, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-HCl, pH 8.0. Resuspended cells were sonicated briefly, and centrifuged at 12,000 x g for 30 minutes. The supernatant fraction was passed over a 5 ml Ni-NTA agarose column (Qiagen, Hilden, Germany) 4-6 times at a speed of 2 ml per minute.

Non-specifically bound protein was removed by washing with both 10 mM and 25 mM imidazole in 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-HCl, pH 8.0. Tumstatin protein was eluted from the column with increasing concentrations of imidazole (50 mM, 125 mM, and 250 mM) in 8 M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-HCl, pH 8.0. The eluted protein was dialyzed twice against PBS at 4°C. A portion of the total protein precipitated during dialysis. Dialyzed protein was collected and centrifuged at approximately 3,500 x g and separated into insoluble (pellet) and soluble (supernatant) fractions.

Please replace the table at page 150, lines 2 through 8 with the following table:

Peptide	Length (in amino acids)	Location within Tumstatin	Sequence
T1	19	1-19	GLKGKRGDSGSPATWTTRG
T2	20	53-72	NQRAHGQDLGTLGSCLRFT
T3	20	68-87	LQRFTTMPFLFCNVNDVCNF
T4	20	83-102	DVCNFA SRNDYSYWLSTPAL
T5	19	98-116	STPALMPMNMAPITGRALE
T6	19	113-131	RALEPYISRCTVCEGPAIA

Please replace the paragraph at page 154, lines 11 through 13 with the following paragraph:

1-12 in "Construct" column refers to the twelve cysteine residues located within full-length Tumstatin, at amino acid positions 34, 67, 79, 85, 122, 125, 144, 178, 190, 196, 236, 239.

Please replace the heading at page 156, line 6 with the heading below, which is marked Example 42. Expression and Purification of Tumstatin-45-132 and Tum-5-125-C-A.

Please replace the paragraph at page 156, lines 19 through 22 with the following paragraph:

Tum-5-125-C-A (SEQ ID NO:34) was made by site-directed mutagenesis of residue 125 (of full-length Tumstatin) from cysteine to alanine, to enhance secretion of Tumstatin-45-132. It was expressed in *E. coli*, and was detected at the same molecular weight size with western blotting using anti-polyhistidine tag antibody.

Please replace the paragraph at page 156, line 23 through page 157, line 11 with the following paragraph:

Goodpasture syndrome is an autoimmune disease characterized by pulmonary hemorrhage and/or rapidly progressing glomerulonephritis, which are caused by the disruption of glomerular and alveolar basement membranes through immune injury associated with autoantibody activity against  $\alpha 3(\text{IV})\text{NC1}$ . Recently, the most probable disease-related pathogenic epitope was identified in the N-terminal portion (Kalluri, R. *et al.*, 1996, *J. Biol. Chem.* 271:9062-8; Hellmark, T. *et al.*, 1999, *Kidney Int.* 55:938-44), and was then further confined within the N-terminal 40 amino acids (Hellmark, T. *et al.*, 1999, *J. Biol. Chem.* 274:25862-8; Netzer, K.O. *et al.*, 1999, *J. Biol. Chem.* 274:11267-74). The N-terminal Tumstatin-45-132 consists of residues 45-132 of Tumstatin, which is outside of the Goodpasture autoepitope. To further confirm that Tumstatin-45-132 would not be detected by Goodpasture autoantibody, antisera from patients with Goodpasture was used for western blotting. This antisera detected 293 cell-expressed full-length Tumstatin with high sensitivity, but failed to detect either *E. coli*-expressed Tumstatin-45-132 and *Pichia*-expressed Tum-5-125-C-A. This shows that Tumstatin-45-132 and Tum-5-125-C-A do not contain the Goodpasture autoepitope, and excludes the

possibility that these recombinant proteins induce this autoimmune disorder upon administration in humans.

Please replace the heading at page 157, line 12 with the following heading:

Example 43. Activities of Tumstatin-45-132 and Tum-5-125-C-A.

Please replace the paragraph at page 158, lines 3 through 13 with the following paragraph:

The effect of Tumstatin-45-132 and Tum-5-125-C-A on the cell cycle were assayed similarly to Example 4 above. Briefly, C-PAE cells were growth arrested by contact inhibition for 48 hours. The cells, at  $10^5$  cells per well, were then harvested and plated into a 12-well plate coated with fibronectin in 5% FCS and either recombinant Tumstatin-45-132 or Tum-5-125-C-A. After 21 hours, the cells were harvested and fixed in 70% ice-cold ethanol. The fixed cells were rehydrated a room temperature for 30 minutes in PBS containing 2% FCS and 0.1% Tween-20, centrifuged and resuspended in 0.5 ml of the same buffer. RNase (5 $\mu$ g/ml) digestion was done at 37°C for one hour, followed by staining with propidium iodide (5  $\mu$ g/ml). The cells were then counted using an EPICS XL-MCL flow cytometer (Beckman-Coulter Instruments, Fullerton, California, USA).

Please replace the paragraph at page 159, lines 15 through 25 with the following paragraph:

Fig. 51 is a histogram showing G<sub>1</sub> arrest of proliferating endothelial cells. In the growth-arrested, contact-inhibited cells, 5.8% of the cells were in S phase at 0 hour. When the cells were stimulated with 5% FCS for 21 hours, there was a 3.7-fold increase



in the percentage of cells in S phase, to 21.5%. Treatment with Tumstatin-45-132 decreased the percentage of cells in S phase to 6.0%. This effect was dose-dependent, with the percentage of cells in S phase being 19.3% at 1  $\mu$ g/ml Tumstatin-45-132, and 11.3% at 10  $\mu$ g/ml Tumstatin-45-132. In another experiment, the percentage of cells in G<sub>0</sub>/G<sub>1</sub> phase was lowest in the 5% FCS-treated control group, and was elevated with treatment with Tumstatin-45-132. These results show that treatment with Tumstatin-45-132 causes cell cycle arrest in proliferating endothelial cells. Treatment with Tum-5-125-C-A showed results equivalent to treatment with Tumstatin-45-132.

Please replace the paragraph at page 159, lines 26 through page 160, line 6 with the following paragraph:

Figs. 52A, 52B, 52C and 52D are a set of four histograms showing the effects of Tumstatin-45-132 and Tum-5-125-C-A on cell viability. Fig. 52A shows cell viability as measured at OD<sub>562</sub> (y-axis) in an MTT assay, for C-PAE cells treated with 0, 3, 6, 12, 25 and 50  $\mu$ g/ml (x-axis) Tumstatin-45-132 (black bars) and Tumstatin-45-132 that was alkylated and reduced (white bars). Tumstatin-45-132 significantly decreased cell viability in a dose-dependent manner with an ED<sub>50</sub> of 12  $\mu$ g/ml. Reduced and alkylated Tumstatin and Tumstatin-45-132 exhibited effects similar to that of non-treated Tumstatin and Tumstatin-45-132 in decreasing cell viability of C-PAE cells. The anti-angiogenic effects of Tumstatin and Tumstatin-45-132 are therefore not dependent on their conformation as derived from disulfide bonds between cysteine residues.

Please replace the paragraph at page 160, lines 7 through 10 with the following paragraph:

Tum-5-125-C-A exhibited effects in cell viability similar to those of Tumstatin-45-132, as shown in Fig. 52B. Fig. 52B shows cell viability as measured at OD<sub>562</sub> (y-

axis) in an MTT assay, for C-PAE cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tum-5-125-C-A.

Please replace the paragraph at page 160, lines 11 through 17 with the following paragraph:

The effects of Tumstatin-45-132 and Tum-5-125-C-A on cell viability of C-PAE cells were not seen in control PC-3 and DU-145 cells, as shown in Figs. 52C and 52D. Fig. 52C shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for PC-3 cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132. Fig. 52D shows cell viability as measured at  $\text{OD}_{562}$  (y-axis) in an MTT assay, for DU-145 cells treated with 0, 3, 6, 12, 25 and 50  $\mu\text{g/ml}$  (x-axis) Tumstatin-45-132. The activity of Tumstatin-45-132 is therefore specific to endothelial cells.

Please replace the paragraph at page 164, line 15 through page 165, line 3 with the following paragraph:

Tumstatin-45-132 was also tested for its ability to suppress tumor growth. Male athymic nude NCRNU mice, of 5-6 weeks of age and about 25 g, were implanted with approximately  $2 \times 10^6$  PC-3 (prostate cancer carcinoma) cells into the dorsal subcutis. The tumors were measured using Vernier calipers and the volume of the tumors calculated using the standard formula ( $\text{width}^2 \times \text{length} \times 0.52$ ). The tumors were allowed to grow to about  $50 \text{ mm}^3$ , and animals were then pair-matched into groups of 6 mice. Initial doses of protein or vehicle (PBS, control) were given on the day of pair-matching (Day 1). Tumstatin-45-132, Tum-5-125-C-A, or human endostatin in sterile PBS was intraperitoneally injected daily b.i.d. at doses ranging from 1 to 20 mg/kg for 20 days. Control animals received injection of PBS vehicle. In one treatment, continuous subcutaneous delivery of Tumstatin-45-132 was done using surgically implanted Alzet

mini-pumps. The mice were weighed twice weekly, and tumor measurements were taken, starting on Day 1. Estimated mean tumor volumes were calculated, and at Day 21, the mice were weighed, sacrificed, and their tumors excised and examined by light microscopy and CD31 immunostaining. The mean treated tumor weight was divided by the mean control tumor weight was subtracted from one, and expressed as a percentage to give the tumor growth inhibition for each group.

Please replace the paragraph at page 165, lines 4 through 17 with the following paragraph:

The results are shown in Fig. 54, which is a line graph showing the fractional tumor volume (y-axis) in terms of  $V/V_0$  (mean tumor volume/initial tumor volume) at 0, 5, 10, 15 and 20 days (x-axis) of treatment with vehicle (control,  $\square$ ), 1 mg/kg Tumstatin-45-132 ( $\blacklozenge$ ), 1 mg/kg Tum-5-125-C-A ( $\bullet$ ), 20 mg/kg endostatin ( $\circ$ ) and mini-pump administered Tumstatin-45-132 (1 mg/kg,  $\Delta$ ). No toxicity from the protein treatments was seen, as judged by weight change. Both Tumstatin-45-132 and Tum-5-125-C-A significantly inhibited the growth of PC-3 cells. Human Tumstatin-45-132 at 1 mg/kg had a tumor growth inhibition of 74.1% ( $p = 0.02$ ) and Tum-5-125-C-A had a tumor growth inhibition of 92.0% ( $p = 0.001$ ), as compared to the vehicle-injected control group. Continuous delivery of Tumstatin-45-132 (1 mg/kg over 24 hours) via an Alzet mini-pump also showed significant tumor growth inhibition of 70.1% ( $p = 0.03$ ). Endostatin delivered at a dose of 20 mg/kg (b.i.d., bolus injection) showed no significant tumor growth inhibition compared to the vehicle-treated control group.

Please replace the paragraph at page 166, lines 5 through 10 with the following paragraph:

Tumstatin-45-132 intraperitoneal injection significantly inhibited microvessel density in PC-3 xenografts as compared to the vehicle-injected control group. The number of CD31-positive blood vessels per low power (40x) field was  $6.33 \pm 0.54$  for Tumstatin-45-132 treatment, versus  $9.44 \pm 1.05$  for the control ( $p = 0.047$ ). Groups treated with Tum-5-125-C-A or the mini-pump-administered Tumstatin-45-132 showed similar decreases of mean vessel density.

Please replace the paragraph at page 170, lines 15 through 25 with the following paragraph:

The potential capacity of tumstatin to inhibit protein synthesis in multiple endothelial cells was therefore explored. Tumstatin and its active subfragments, Tumstatin-45-132, T3 and T7 peptides were used. The amino acids 45-132 of Tumstatin were expressed as recombinant Tumstatin-45-132 in *E. coli* as described above and in (Maeshima, Y. *et al.*, 2001, *J. Biol. Chem.* 276:15240-8). Human endostatin was produced in yeast as described in (Dhanabal, *et al.*, 1999, *Cancer Res.* 59:189-97). Only soluble protein with a low endotoxin level (less than 50 EU/mg) was used. T3 peptide, T7 peptide, consisting of residues 68-87 and 73-97 of tumstatin, respectively, and T7-mutant peptide (TMPFMFCNINNVCNFA SRNDYSYWL; SEQ ID NO:38) were synthesized as described in (Maeshima, Y. *et al.*, 2000, *J. Biol. Chem.* 275:21340-8; Maeshima, Y. *et al.*, 2001, *J. Biol. Chem.* 276:31959-68).

Amendments to the specification are indicated in the attached "Marked Up Version of Amendments" (pages i - xxii).

#### In the Claims

Please amend claims 43, 48 and 49 as follows. Amendments to the claims are indicated in the attached "Marked Up Version of Amendments" (pages xxiii - xxv).